The effects of hyaluronic acid on articular chondrocytes

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The purpose of this study was to examine the effects of hyaluronic acid supplementation on chondrocyte metabolism in vitro. The clinical benefits of intra-articular hyaluronic acid injections are thought to occur through improved joint lubrication. Recent findings have shown that exogenous hyaluronic acid is incorporated into articular cartilage where it may have a direct biological effect on chondrocytes through CD44 receptors.

Bovine articular chondrocytes were isolated and seeded into alginate constructs. These were cultured in medium containing hyaluronic acid at varying concentrations. Samples were assayed for biochemical and histological changes.

There was a dose-dependent response to the exposure of hyaluronic acid to bovine articular chondrocytes in vitro. Low concentrations of hyaluronic acid (0.1 mg/mL and 1 mg/mL) significantly increase DNA, sulphated glycosaminoglycan and hydroxyproline synthesis. Immunohistology confirmed the maintenance of cell phenotype with increased matrix deposition of chondroitin-6-sulphate and collagen type II. These findings confirm a stimulatory effect of hyaluronic acid on chondrocyte metabolism.

Therapeutic efforts to treat osteoarthritis are usually directed towards providing symptomatic relief and delaying the need for total joint arthroplasty. Recently, treatments such as glucosamine and chondroitin sulphate supplementation, intra-articular injection of hyaluronic acid, subchondral drilling, mosaic-plasty, tissue engineered focal defect repair and autologous chondrocyte implantation are examples of a shift in emphasis from simple analgesia to preventative approaches.

Synovial fluid from osteoarthritic joints has a much lower elasticity and viscosity than that from normal joints. This decrease in the rheological properties of synovial fluid results from both a reduced molecular size and a reduced concentration of hyaluronic acid in the synovial fluid. Intra-articular injections of hyaluronic acid, or its derivatives, are used in an attempt to return the elasticity and viscosity of the synovial fluid to normal.

Clinical studies have shown that hyaluronic acid injections are well tolerated and provide sustained relief from pain and functional disability.

The physical properties of hyaluronic acid are important but there is evidence to suggest that hyaluronic acid may provide both physiochemical and pharmacological advantages. Chondrocytes express the glycoprotein CD44 on their cell surface; this has the capacity to function as a hyaluronic acid receptor and so may be involved in biochemical interactions with chondrocytes. The effects of hyaluronic acid injections may be mediated via CD44 interactions. Exogenous hyaluronic acid is known to be incorporated into cartilage.

The purpose of this study was to determine the in vitro effects of hyaluronic acid supplementation on articular chondrocyte activity and their ability to synthesise essential components of the articular cartilage extracellular matrix. The effects of hyaluronic acid on chondrocyte proliferation, measured by DNA production, sulphated glycosaminoglycan (GAG) synthesis, collagen type II synthesis and maintenance of phenotype was determined. This study also evaluated the possibility of supplementing hydrogels with hyaluronic acid to improve the quality of the gel/chondrocyte constructs for the purpose of tissue-engineered repair of focal articular cartilage defects.

Materials and Methods

Bovine articular chondrocytes were cultured on alginate beads containing various concentrations of hyaluronic acid for a period of 14 days with a regular change of hyaluronic acid-containing medium. DNA, sulphated GAG and collagen synthesis in the culture specimens.
were quantified using well-established assaying techniques. Chondrocyte proliferation and extra cellular matrix production were determined by histological assessment using haematoxylin and eosin staining. The immunolocalisation of chondroitin-6-sulphate and collagen types I and II was also performed to assess the quality of the extracellular matrix.

Isolation of chondrocytes. Bovine articular cartilage was harvested from the metacarpophalangeal joints of six 18-month-old calves. The cartilage was obtained aseptically within six hours of slaughter and the entire cartilage from each joint was removed. Extracted articular cartilage slices were placed immediately into Earl’s Balanced Salt Solution (EBSS) (Sigma, Poole, UK). The tissues were finely diced and washed in EBSS. Cells were released from the tissue by sequential enzyme digestion, firstly in 10 mg/mL pronase E (Merck, Poole, UK) in Dulbecco’s Modified Eagle’s Medium (DMEM) (Life Technology, Paisley, UK) + 20% Fetal Calf Serum (FCS) (Life Technology) at 37˚C on a rolamixer. The supernatant containing released chondrocytes was then filtered through a sterile 70 µm nylon sieve (Falcon, Oxford, UK) to remove undigested cartilage, washed twice in DMEM + 20% FCS and finally resuspended in culture medium (DMEM, supplemented with 20% FCS, 1 mM HEPES buffer, 1% penicillin/streptomycin, 1% L-glutamine and 50 µg/mL L-ascorbic acid). The number of cells and their viability was determined by trypan blue staining and a haemacytometer.

Cell culture. The isolated cells were resuspended in 2% low-viscosity alginate hydrogel (Kelco Neutrosweet, Tadworth, UK) containing Rooster comb hyaluronic acid (Sigma) at a density of 5 x 10^6 cells/mL. The alginate gels were made to four different concentrations of hyaluronic acid: 0.1, 1.0, 2.0, and 3.0 mg/mL. The control specimens contained alginates that was free from hyaluronic acid.

The alginate/chondrocyte/hyaluronic acid suspensions were then gently expressed through a 22-gauge needle attached to a 5 mL syringe into a 100 mM CaCl_2 solution in a sterile Petri dish and each drop polymerised to form an alginate bead containing approximately 100 000 chondrocytes (Syringe droplet technique). Twenty minutes of incubation was allowed for further polymerisation. The newly-formed beads were then washed twice in EBSS to remove excess CaCl_2. Five sets of beads containing hyaluronic acid at the four different concentrations, and a control set of beads containing chondrocytes without hyaluronic acid, were formed and were used for the culture experiments.

Each set of alginate beads was then transferred into sterile 24-well culture plates, with eight randomly chosen beads placed in each well containing 1 mL of culture medium supplemented with hyaluronic acid at the same concentration as that in the respective alginate beads. The hydrogel constructs were cultured for a period of 14 days in the humidified atmosphere of a standard incubator at 37˚C and 5% CO₂. The culture medium was replaced by fresh culture medium every three days.

Biochemical analysis. Six replicates of eight alginate beads were removed randomly from each of the five cultures at 0, 3, 6, 9, and 14 days for analysis. In order to release the cells and matrix from alginate, the chondrocyte/alginate beads were dissolved in a buffer containing 55 mM sodium citrate (BDH, Poole, UK), 150 mM sodium chloride (BDH), 5 mM cysteine hydrochloride (Sigma), 5 mM EDTA (BDH) and 0.56 mg/mL of papain (Sigma), at 60˚C for 24 hours.

The total DNA was determined using the Hoechst method described by Rao and Otto and Rago, Mitchen and Wilding. Calf thymus DNA, in a 1:1 solution of saline sodium citrate + 0.4% (v/v) sodium dodecylsulphate and papain digest buffer, was used for preparation of the standard dilution range from 0 to 20 µg/mL. We added 100 µL aliquots of standards or samples to appropriate wells in a fluorimetric well plate. We also added 100 µL Hoechst 33258 (1 µg/mL) in saline sodium citrate to each well and the plates were read on a fluorimeter (Fluoroskan Ascent, LabSystems, Cheshire, UK) with a 365 nm excitation filter and emission set at 460 nm.

The measurement of total sulphated GAG in the chondrocyte cultures was performed using the 1,9-dimethylmethylene blue (DMB) dye method, which had been modified for use with alginate systems at a pH of 1.5. Chondroitin sulphate A from shark fin (Sigma) was diluted with distilled water to prepare a standard curve (0 to 100 µg/mL). We added 40 µL of the papain-digested samples or GAG standards to 250 µL of DMB solution in wells of an absorbance plate. GAG content was determined using a spectrophotometer (Biorad Plate-Reader, Bio-Rad Laboratories, Hemel Hempstead, UK) at a wavelength of 595 nm.

The measurement of the hydroxyapatite content of the alginate beads was performed using an oxidation assay which was run in the Cobas Bio centrifugal analyser (Roche, Rotkreuz, Switzerland) at 40˚C. The assay involves the oxidation of the amino acid hydroxyproline in the samples by chloramine T. The chromogens formed (pyrrole and peryl-2-carboxylic acid) are coupled with p-dimethylaminobenzaldehyde (DMAB) to produce a chromophore whose absorbance can be measured at 570 nm. Standard curves were used to determine the hydroxyproline concentrations in the sample range 0 to 100 µmol L⁻¹.

Histological analysis. Specimens were collected on days 0, 3, 6, 9 and 14 for the five alginate bead sets and were fixed at 4˚C in buffer consisting of 4% paraformaldehyde (AGAR Scientific Ltd, Stansted, UK), 100 mM sodium cacodylate (AGAR Scientific Ltd) and 10 mM CaCl_2 (BDH) at pH 7.4 for 24 hours. Samples were then washed overnight at 4˚C in 0.1 M sodium cacodylate and 50 mM BaCl_2 (Merck) at pH 7.4. The fixed beads were then dehydrated through a series of ethanol concentrations, cleared with xylene and embedded in paraffin wax. Six sections were cut at a thickness of 5 µm, using a sledge microtome, mounted onto glass slides, rehydrated and deparaffinised.
The sections were stained with Harris’s acid alum haematoxylin and eosin in order to demonstrate cell nuclei and extracellular matrix. The sections were mounted in a synthetic mounting medium (DPX - dibutylphthalate + xylene) for microscopic examination (Model, BH2, Olympus, UK). Immunolocalisation for collagen types I and II and GAG chondroitin-6-sulphate. Immunolocalisation of the chondrocyte-seeded alginate beads for chondroitin-6-sulphate and collagen was performed using the streptavidin biotin-immunoperoxidase method. This process was also used for detection of the Ki-67 nuclear antigen. This is a nuclear antigen found only in proliferating cells and gives a reliable indication of the presence of healthy replicating chondrocytes. Paraffin wax sections of the cell/alginate construct were initially de-waxed in xylene, then soaked in an endogenous peroxidase block consisting of 3% (v/v) \( \text{H}_2\text{O}_2 \) in methanol for 15 minutes, followed by pre-treatment with a trypsin solution (0.1% (w/v) trypsin (Sigma) and 0.1% (w/v) \( \text{CaCl}_2 \) in distilled water) at 37°C for ten minutes. In contrast, the sections to be stained for chondroitin-6-sulphate were pre-treated with chondroitinase ABC (0.125 IU/mL) for 60 minutes. The sections were then blocked with rabbit serum (DakoCytomation Ltd, Cambridgeshire, UK) diluted 1:5 in Tris Buffered Saline (TBS) (0.05 m TBS, 0.15 m sodium chloride, pH 7.6) for 20 minutes.

Subsequently, the sections were incubated with the relevant primary antibody, which consisted of CIICI (DSHB, University of Iowa, Iowa) diluted 1:2 with TBS, Col-1 (Sigma) diluted 1:100 and 3-B-3 (ICN/FLOW) diluted 1:100 to stain for collagen type II, collagen type I and chondroitin-6-sulphate, respectively. The primary antibody was then removed by washing in TBS for five minutes, and incubated for 30 minutes at room temperature with biotinylated rabbit anti-mouse secondary antibody (Dako) 1:200 in TBS. The sections were then washed and incubated with streptavidin/biotin/horseradish peroxidase complex (Dako Cytomation, Glostrup, Denmark) for 30 minutes at room temperature. They were then rinsed and incubated with DAB peroxidase solution (Sigma) for ten minutes. The samples were counterstained with Harris’s haematoxylin for two minutes and mounted with DPX, a mixture of distyrene, tricresyl phosphate and xylene.

**Statistical analysis.** Graphs were plotted to represent the mean (SEM) of six replicate samples. Two-way analysis of variance was carried out to assess the effects of dose, day and the statistical interaction between these two factors. Post hoc Dunnet’s T3 tests were carried out to assess the difference between each dose and the control (zero) dose. Checks on assumptions of normality and constant variance were also performed. Assumptions of constant variance held for GAG and hydroxyproline data, but did not hold for DNA values. Therefore, analysis was repeated on log-transformed DNA values, but gave essentially similar results. Consequently, analyses on untransformed data have been reported.

**Results**

Our results showed that articular chondrocytes cultured in the presence of hyaluronic acid have a significantly greater rate of DNA proliferation and extracellular matrix production, compared with chondrocytes cultured without hyaluronic acid. Cell proliferation and matrix synthesis depended upon the concentration of hyaluronic acid present, with the greater cellular responses being seen at lower hyaluronic acid concentrations.

**Table I. Mean DNA content (µg/mL) of cell-seeded alginate bead constructs cultured in medium containing varying concentrations of hyaluronic acid for up to 14 days**

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<th>Day</th>
<th>Control</th>
<th>0.1 mg/ml</th>
<th>1.0 mg/ml</th>
<th>2.0 mg/ml</th>
<th>3.0 mg/ml</th>
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Biochemistry. The chondrocytes exposed to 0.1 µg/mL hyaluronic acid showed the greatest levels of DNA proliferation and sulphated GAG and collagen production. Significantly higher levels of DNA and extracellular matrix components were recorded on days 9 and 14 in the cultures exposed to both 0.1 µg/mL and 1.0 µg/mL hyaluronic acid compared with the controls (p = 0.01). As the concentration of hyaluronic acid was increased to 2.0 and 3.0 mg/mL, the level of DNA and extracellular matrix synthesis declined considerably, although it remained higher than in the controls.

DNA analysis. The DNA content of the control and hyaluronic acid-treated chondrocyte groups is shown in Figure 1 and Table I. An overall increase in DNA content occurred in all study groups during the culture period, which indicated an increase in cell numbers. In all conditions there appeared to be an initial decrease in DNA content during the first three days of culture. At days 9 and 14 there was a statistically significant mean increase in content for cultures exposed to 0.1 and 1.0 µg/mL hyaluronic acid (p = 0.01). As the concentration of hyaluronic acid was increased to 2.0 and 3.0 mg/mL, the level of DNA and extracellular matrix synthesis declined considerably, although it remained higher than in the controls.

Sulphated GAG analysis. The GAG content of the control and hyaluronic acid-treated chondrocyte groups is shown in Figure 2 and Table II. Again, a significant interaction was observed between day and dose (p < 0.001); the effect of all doses, particularly at 0.1 mg/mL and 1.0 mg/mL, was seen more clearly for days 9 and 14 when compared with the earlier timepoints. There was an overall increase in GAG content within the alginate beads in all five cultures. The increase in GAG content was greater in all chondrocyte cultures exposed to hyaluronic acid compared with the control group and there was a dose-dependent response to hyaluronic acid. A steady increase in GAG content in all culture groups occurred up to day six, the content between the groups being very similar. At days 9 and 14 the mean GAG content in chondrocytes cultured in 0.1 mg/mL and 1.0 mg/mL hyaluronic acid was significantly greater (p = 0.005) than the control group. The largest increase (307%), occurred in the 0.1 mg/mL hyaluronic acid group. At hyaluronic acid concentrations of 2.0 mg/mL and 3.0 mg/mL no significant difference in GAG content was found when compared with the control groups.

Hydroxyproline analysis. The level of hydroxyproline is a marker for collagen production by the chondrocytes. Figure 3 and Table III show the concentration of hydroxyproline in the chondrocyte/alginate composite beads exposed to the different concentrations of hyaluronic acid. All the culture groups demonstrated a significant increase by day 14 (p = 0.007) compared with day 0. A significant interaction was observed between day and dose (p = 0.003); the effect of doses at 0.1 mg/mL and 1.0 mg/mL hyaluronic acid were, once again seen more clearly for days 9 and 14 than for earlier days. The mean increases in hydroxyproline concentration were significantly greater in the chondrocytes exposed to 0.1, 1.0, and 2.0 mg/mL of hyaluronic acid compared with the control groups (p = 0.03) at days 9 and 14. The increase in hydroxyproline concentration in the hyaluronic acid groups demonstrated a dose-dependent response, with doses greater than 0.1 mg/mL hyaluronic acid showing a smaller increase in hydroxyproline.

Histology and immunolocalisation. Histology demonstrated changes in cellular morphology, behaviour and extracellular matrix.
lar matrix that were consistent with the biochemical findings. As a marker for cell proliferation, Ki-67 antigen immunostaining was strongly positive in the low-dose hyaluronic acid groups at day 14.

There was increased cellularity in the 0.1 mg/mL hyaluronic acid (low-dose) and 3 mg/mL hyaluronic acid (high-dose) cultures compared with the control. Greatest cellularity occurred in the 0.1 mg/mL hyaluronic acid specimens. The cells were either actively dividing or were present in small clusters of three to four cells. In the 3 mg/mL hyaluronic acid specimens, the cells had divided but fewer clusters were identified compared with the low-dose hyaluronic acid specimens. The results from the 2 mg/mL hyaluronic acid specimens showed intermediate findings. The majority of cells in the control group remained singular with very few mitotic figures.

The quality of the synthesised extracellular matrix was assessed by immunolocalisation of key articular cartilage matrix components. Increased levels of chondroitin-6-sulphate and collagen type II staining were present within culture specimens containing hyaluronic acid, compared with the controls. The presence of extracellular matrix rich in type II collagen and chondroitin-6-sulphate, combined with the absence of type I collagen, indicates that the chondrocytic phenotype was maintained throughout the culture period. Although the control specimens showed small increases in these components, the levels were by no means as marked as for the hyaluronic acid cultures. The most intense staining for collagen type II and chondroitin-6-sulphate was seen within beads containing 0.1 mg/mL and 1 mg/mL. Hyaluronic acid staining was less within beads containing 2 mg/mL and 3 mg/mL hyaluronic acid, although all were greater than the control beads.

The detection of chondroitin-6-sulphate indicated the presence of sulphated GAG. There was intense chondroitin-6-sulphate immunostaining around chondrocytes (in the territorial matrix) cultured in low-dose hyaluronic acid. Staining around the cells cultured in the control group, and with high concentration of hyaluronic acid, was less intense at day 14. Cells cultured in low concentrations of hyaluronic acid also stained more intensely for collagen type II. All chondrocytes cultured with hyaluronic acid stained negatively for collagen type I, whereas there was collagen type I staining within the control group.

Discussion
The purpose of this study was to investigate the effect of hyaluronic acid on articular chondrocytes cultured in vitro using a hydrogel culture system.

The rationale behind the treatment of early osteoarthritis with intra-articular hyaluronic acid injections has been viscosupplementation therapy, which attempts to restore the elasticity and viscosity of synovial fluid to normal or higher levels.\(^8\) Pharmokinetic studies have shown that exogenous hyaluronic acid begins to leave the joint cavity within two hours of administration and is cleared completely within four days, but also showed that a fraction of the hyaluronic acid remains detectable in articular cartilage six hours after administration.\(^9\) The short duration of hyaluronic acid within the joint does not fully explain the indisputable long-term clinical efficacy seen in practice and appears to reject the proposed mechanism of action of exogenous hyaluronic acid as purely one of viscosupplementation. Hyaluronic acid possesses a number of protective physiochemical functions that may provide some additional chondroprotective effects in vivo and may explain its longer term effects on articular cartilage. These functions include scavenging of reactive oxygen-derived...
free radicals, inhibition of immune complex adherence to polymorphonuclear cells, inhibition of leucocyte and macrophage migration and aggregation, and regulation of fibroblast proliferation.

We found that at low doses, hyaluronic acid has a significant stimulatory effect on the metabolic activity of chondrocytes that may provide an explanation for the longer term clinical benefits. Studies on osteoarthritic cartilage have shown that hyaluronic acid depletion in the extra-cellular matrix occurs before structural changes in proteoglycans are detectable. It is likely that physiochemical interactions occur between hyaluronic acid and chondrocytes which regulate their activity and ability to produce proteoglycans and type II collagen.

The recent identification of CD44, a glycoprotein expressed on the cell surface of chondrocytes, may help explain the method of interaction of hyaluronic acid with chondrocytes. CD44 has the capacity to function as a hyaluronic acid receptor at the chondrocyte cell surface. We found a dose-dependent response that could be attributed to a negative feedback system via CD44 receptors, whereby chondrocyte proliferation and extracellular matrix production is increased at low concentrations and decreased at high concentrations of hyaluronic acid. Our results are consistent with the findings of Kawasaki et al who, in collagen gels, found that cell numbers were greatest on exposure to low doses of 0.1 mg/mL hyaluronic acid, followed by 1.0 mg/mL hyaluronic acid.

If low concentrations of hyaluronic acid have a stimulatory effect on chondrocytes, this should occur in articular cartilage with low indigenous hyaluronic acid concentrations, such as in osteoarthritic cartilage. Despite the naturally low hyaluronic acid concentration, the structural progression of osteoarthritis appears to continue. There may be a number of possible factors that explain this paradox. This study used healthy bovine articular chondrocytes and it is possible that in vivo, osteoarthritic human articular chondrocytes have a reduced level of interaction with their surrounding extracellular matrix, perhaps as a result of down-regulation of CD44 receptor expression, and thus a reduced level of response to hyaluronic acid. Further studies are needed to investigate this.

Our study has also demonstrated the potential of using the alginate/chondrocyte/hyaluronic acid construct as a cartilage transplant biomaterial for the repair of articular cartilage defects. Chondrocytes maintained their phenotype and proliferated and synthesised extracellular matrix within the construct. A previous problem with alginate was a time-dependent leakage of hyaluronic acid from the scaffold so that sufficient hyaluronic acid retention could only be achieved in alginate gels with densities greater than 1.2%. In our study the alginate gel density used was 1.0 mg/mL hyaluronic acid which may provide an explanation for the longer term clinical benefits. The same concentration as that in the alginat bead in order to address the problem of hyaluronic acid diffusion out of the alginate gel. This was to improve the diffusion of nutrients into the alginate from the medium and to allow sustained stimulatory effects of hyaluronic acid on the chondrocytes.

A recent study assessed the effect of hyaluronic acid on chondrocytes cultured in collagen gel as a potential construct and also found chondrocyte proliferation and GAG synthesis to be enhanced. Although chondrocyte proliferation was greater in the collagen gels, chondrocytes dedifferentiated into fibroblast-like cells after six days in culture. Chondrocytes cultured in alginate, however, maintained their phenotype.

Alginate has been proposed as a suitable biomaterial for a construct in which chondrocytes can be embedded, cultured and transplanted into cartilage defects. We have shown that supplementation of hyaluronic acid into alginate to create an alginate/chondrocyte/hyaluronic acid construct could be a more suitable transplant system for cartilage repair including tissue-engineered repair of focal articular cartilage defects.

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No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

References


